Please cite this article in press as: Yu et al., Dynamic Phosphorylation of CENP-A at Ser68 Orchestrates Its Cell-Cycle-Dependent Deposition at Centromeres, Developmental Cell (2015), http://dx.doi.org/10.1016/j.devcel.2014.11.030

Developmental Cell
Article

Dynamic Phosphorylation of CENP-A at Ser68 Orchestrates Its Cell-Cycle-Dependent Deposition at Centromeres

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http://dx.doi.org/10.1016/j.devcel.2014.11.030

SUMMARY

The H3 histone variant CENP-A is an epigenetic marker critical for the centromere identity and function. However, the precise regulation of the spatiotemporal deposition and propagation of CENP-A at centromeres during the cell cycle is still poorly understood. Here, we show that CENP-A is phosphorylated at Ser68 during early mitosis by Cdk1. Our results demonstrate that phosphorylation of Ser68 eliminates the binding of CENP-A to the assembly factor HJURP, thus preventing the premature loading of CENP-A to the centromere prior to mitotic exit. Because Cdk1 activity is at its minimum at the mitotic exit, the ratio of Cdk1/PP1α activity changes in favor of Ser68 dephosphorylation, thus making CENP-A available for centromeric deposition by HJURP. Thus, we reveal that dynamic phosphorylation of CENP-A Ser68 orchestrates the spatiotemporal assembly of newly synthesized CENP-A at active centromeres during the cell cycle.

INTRODUCTION

The centromere is essential for dividing cells to faithfully propagate their genetic material in equal parts to their daughter cells (Allshire and Karpen, 2008). In most eukaryotes, centromeric DNA sequences seem neither necessary nor sufficient for the identity and function of centromeres (Allshire and Karpen, 2008). Therefore, centromeres are now defined epigenetically, namely, by the presence of CENP-A (also known by its generic name CenH3), a centromere-specific variant of histone H3 (Allshire and Karpen, 2008; Cheeseman and Desai, 2008; Earnshaw and Rothfield, 1985). In contrast to the tight linking of the synthesis and deposition of canonical histones to DNA replication (Marzluff et al., 2008), the processes of CENP-A synthesis and assembly into chromatin are uncoupled from centromeric DNA replication in most species studied to date (Jansen et al., 2007; Schuh et al., 2007; Shelby et al., 2000). Centromeric DNA is replicated in the mid-to-late S phase (Ten Hagen et al., 1990), while mRNA and protein levels of CENP-A peak during the late G_2 and mitotic phases (Shelby et al., 1997, 2000). After DNA replication in the S phase, each newly synthesized centromere inherits about half of the parental CENP-A, which must then be complemented before the next round of DNA replication (Allshire and Karpen, 2008; Nechemia-Arbely et al., 2012). Surprisingly, CENP-A replenishment occurs at distinct time points in the different eukaryotic systems studied. In human cells, chicken DT40 cells, and *Xenopus* egg extracts, the assembly of newly synthesized CENP-A occurs during late telophase and early G_1 phase, a short time window following mitotic exit (Jansen et al., 2007; Moree et al., 2011; Silva et al., 2012). In *Drosophila*, the newly synthesized CENP-A assembles into centromeric chromatin at telophase/early G_1 in somatic tissues (Dunleavy et al., 2012), at metaphase in cultured *Drosophila* cell lines (Mellone et al., 2011), and at anaphase during embryonic syncytial divisions (Schuh et al., 2007). In contrast to the species described above, the assembly of CENP-A in yeast and *Arabidopsis thaliana* has been reported to occur during the S phase or late G_2 phase (Dunleavy et al., 2007; Lermontova et al., 2006).

Recently, a number of studies demonstrated that the assembly of CENP-A chromatin is temporally coupled to cell-cycle-related events, including the activation of the anaphase-promoting complex/cyclosome, the degradation of cyclin A in flies (Erhardt et al., 2008; Mellone et al., 2011), the centromeric loading of chromatin licensing factors, and the inhibition of cyclin-dependent kinase (Cdk) activities in mammalian cells (McKinley and Cheeseman, 2014; Müller et al., 2014; Silva et al., 2012). Prior to deposition, specific factors, including the centromere-licensing factor Mis18 complex as well as RbAp46 and RbAp48 (RbAp46/48), are recruited for the priming of

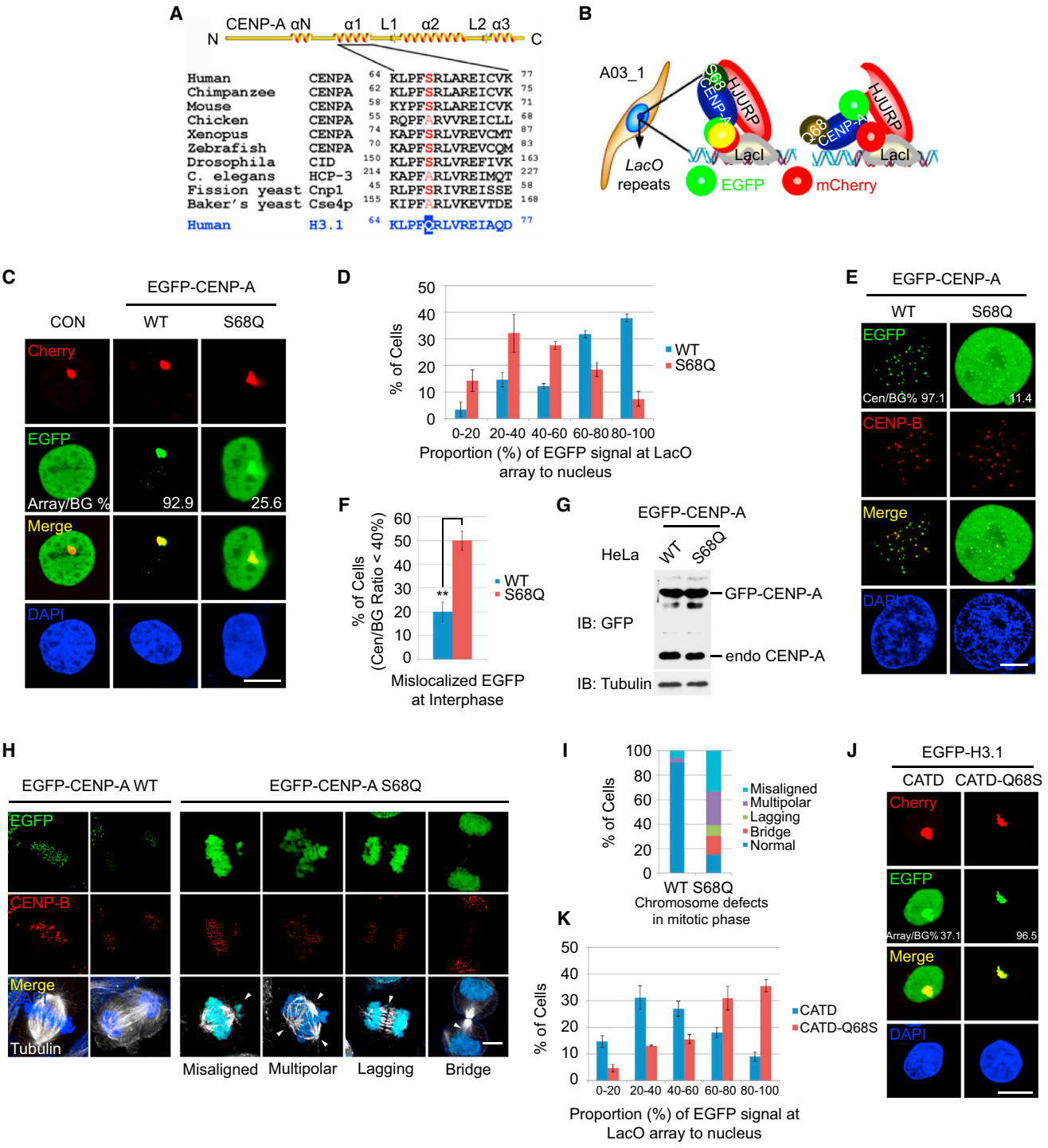

Figure 1. Ser68 Phosphorylation Is Critical for the Functions of CENP-A

(A) Amino acid sequence alignments of the α1-helix of CENP-A homologs in several species. Numbers adjacent to the sequences indicate the position of the residue, counted from the N terminus of each protein. Conserved residues are in red (S or A in CENP-A).

(B) Schematic diagram illustrating the LacI-*LacO* targeting assay used in this study.

(C) Representative images of A03_1 cells transfected with EGFP-tagged CENP-A wild-type (WT) or mutants (green) along with mCherry-LacI-HJURP (red) and stained with DAPI, (for DNA, blue). Cells transfected with EGFP-H3.1 were set as controls. BG, background. Scale bar, 10 μm.

(D) Quantification of cells expressing EGFP-tagged proteins specifically enriched at *LacO* arrays in (C). The enrichment is defined by the ratio (%) of the signal on the array to background signal (nucleus). Data are presented as the mean ± SD of three independent experiments.

(E) Representative images of localization pattern of EGFP-tagged CENP-A WT and the S68Q mutant (green) in HeLa cells. Cells were stained by anti-CENP-B (for centromeres, red) and DAPI (blue) as indicated. Scale bar, 5 μm. Expression of the transfected proteins was analyzed by western blot shown in (G).

(legend continued on next page)

centromeric regions allowing the loading of CENP-A (Fujita et al., 2007; Hayashi et al., 2004; Maddox et al., 2007). In human cells, the deposition of CENP-A is mediated by the specific chaperone and assembly factor HJURP (Dunleavy et al., 2009; Foltz et al., 2009), which itself is recruited there by the Mis18 complex (Barnhart et al., 2011; Fujita et al., 2007).

The centromere-targeting domain (CATD) of CENP-A is required for the binding of HJURP, suggesting a potential role of CATD for centromeric recruitment of CENP-A (Dunleavy et al., 2009; Foltz et al., 2009). Recently, we and other groups revealed the structural basis for the recognition of CenH3^{CENP-A}/CenH3^{Cse4} by HJURP/Scm3 (the HJURP yeast homolog) (Cho and Harrison, 2011; Hu et al., 2011; Zhou et al., 2011). Intriguingly, unlike previous studies that identified CATD as the exclusive region responsible for HJURP binding (Bassett et al., 2012; Black et al., 2004), our structural and biochemical analysis showed that the region outside CATD contains an essential residue for HJURP binding, with the residue being identified as Ser68 (Hu et al., 2011). In order to identify the potential functional significance of Ser68 in vivo, as well as to investigate how CATD and Ser68 cooperate with each other in CENP-A centromeric recruitment, we analyzed the capability of CENP-A Ser68 mutation both for HJURP binding and centromere targeting using biochemical and cellular biology techniques. Furthermore, we generated a phosphorylation-specific antibody directed against Ser68-P, allowing us to identify this residue as a cell-cycle-dependent phosphorylation site, thus revealing its essential physiological role in CENP-A deposition at centromeres.

RESULTS

Ser68 Is Necessary, but Not Sufficient, for the Function of CENP-A In Vivo

CENP-A Ser68 is evolutionarily conserved in most eukaryotes, with the exception of budding yeast, *C. elegans*, and chicken. In contrast, the canonical histone H3.1 carries a glutamine at this site, indicating that this residue represents a potential functional marker for the CENP-A variant form (Figure 1A). To investigate the physiological relevance of this conserved residue in vivo, we generated an S68Q mutation of CENP-A (CENP-A^{S68Q}) that was predicted to produce a steric clash with a hydrophobic notch present in HJURP (Hu et al., 2011). Using this mutant form, we performed in vivo LacI (Lac repressor)-*LacO* (*Lac* operon) targeting (Robinett et al., 1996) and coimmunoprecipitation (co-IP) experiments. It has been reported that LacI-fused HJURP could drive the recruitment of CENP-A to the *LacO* arrays at a noncentromeric locus (Figure 1B) (Barnhart et al., 2011). In our system, multiple *LacO* arrays were integrated into a single noncentromeric chromatin locus in A03_1 Chinese hamster ovary DG44 cells (Qiu et al., 2011). We found that the EGFP-tagged wild-type CENP-A, but not the CENP-A^{S68Q} mutant, was recruited to the *LacO* arrays by an ectopically expressed mCherry-LacI-HJURP fusion protein (Figures 1C and 1D), indicating that the S68Q mutation resulted in impaired binding of CENP-A to HJURP. In addition, the coIP experiments showed that the interaction between CENP-A and HJURP was greatly impaired in S68Q (Figure S1A available online). Using a colorectal cancer cell line HCT-116 carrying knocked-in FLAG-tagged HJURP, we confirmed that the S68Q mutation impaired the binding activity of CENP-A to endogenous HJURP (Figure S1B). To confirm our assumption that Ser68 is essential for HJURP binding, we separated soluble (chromatin-unbound) CENP-A from the chromatin-bound CENP-A using a biochemical fractionation approach. Our results clearly showed that significantly more CENP-A^{S68Q} proteins were observed in the soluble fraction than wild-type CENP-A (Figures S1C and S1D). Thus, our data strongly suggest that, in the CENP-A^{S68Q} mutant form, the binding of CENP-A to HJURP is severely reduced, which directly impairs the recruitment of CENP-A to chromatin.

H3.1^{CATD} is an artificial chimeric histone in which the CATD domain of H3.1 was substituted with the corresponding region of CENP-A, and it has been reported to incorporate into centromere and specify the location for kinetochore as CENP-A (Black et al., 2007; Foltz et al., 2009). This chimera protein exhibited a weak interaction with HJURP in our LacI-*LacO* targeting and coIP experiments (Figures 1J, 1K, S2A, and S2B). Interestingly, we found that the single-point mutation of Q68S in H3.1^{CATD} (H3.1$^{CATD\text{-}Q68S}$) significantly increased binding capacity to HJURP (Figures 1J, 1K, and S2B), suggesting that Ser68 confers an extra CENP-A attribute to H3^{CATD}. However, canonical H3.1 carrying the Q68S mutation (H3.1^{Q68S}) did not exhibit any binding capacity for HJURP, similar to wild-type H3.1 (Figures S2A and S2B), indicating that Ser68 alone is not sufficient for HJURP binding. Together, these results indicate that CATD and Ser68 are structural elements, both of which are essential for centromere recruitment of CENP-A.

To investigate the role of Ser68 in CENP-A function in vivo, we tested the effects of Ser68 mutation on the centromeric localization of CENP-A using immunofluorescent imaging. In contrast to the centromere-specific localization of wild-type CENP-A and H3.1$^{CATD\text{-}Q68S}$, the CENP-A^{S68Q} and H3.1^{CATD} mutants showed genome-wide distributions in the interphase nucleus of HeLa cells (Figures 1E–1G and S2C). The mislocalization of CENP-A mutants resulted in the merging of CENP-A signal with that of the condensed chromosomes in mitotic cells and was associated with various mitotic chromosomal defects, such as aneuploidy, chromosome bridges, and formation of polypolar spindles during the mitotic phase (Figures 1H and S2E).

(F) Quantification of cells with mislocalized EGFP-CENP-A wild-type or S68Q in (E). The mislocalization is defined by the ratio (%) of EGFP signal at the centromere to background signal (nucleus) as indicated. Data are presented as the mean ± SD of three independent experiments. **$p < 0.01$ (Student's t test).
(G) Western blot showing the protein expression of endogenous and exogenous CENP-A in (E).
(H) Representative images of mitotic defects in HeLa cells expressing EGFP-tagged CENP-A S68Q compared to CENP-A WT (green), cells were stained with anti-CENP-B (for centromeres, red) and DAPI (blue) as indicated. Scale bar, 5 μm.
(I) Quantification of cells with different types of chromosome defects shown in (H).
(J) Representative images of A03_1 cells transfected with EGFP-tagged H3.1CATD or H3.1CATD-Q68S (green) along with mCherry-LacI-HJURP (red) and stained with DAPI (blue). Scale bar, 10 μm.
(K) Quantification of cells expressing EGFP-tagged proteins specifically enriched at *LacO* arrays in (J). The enrichment was defined by the ratio (%) of the signal on the array to in the background (nucleus). Data are presented as mean ± SD of three independent experiments.

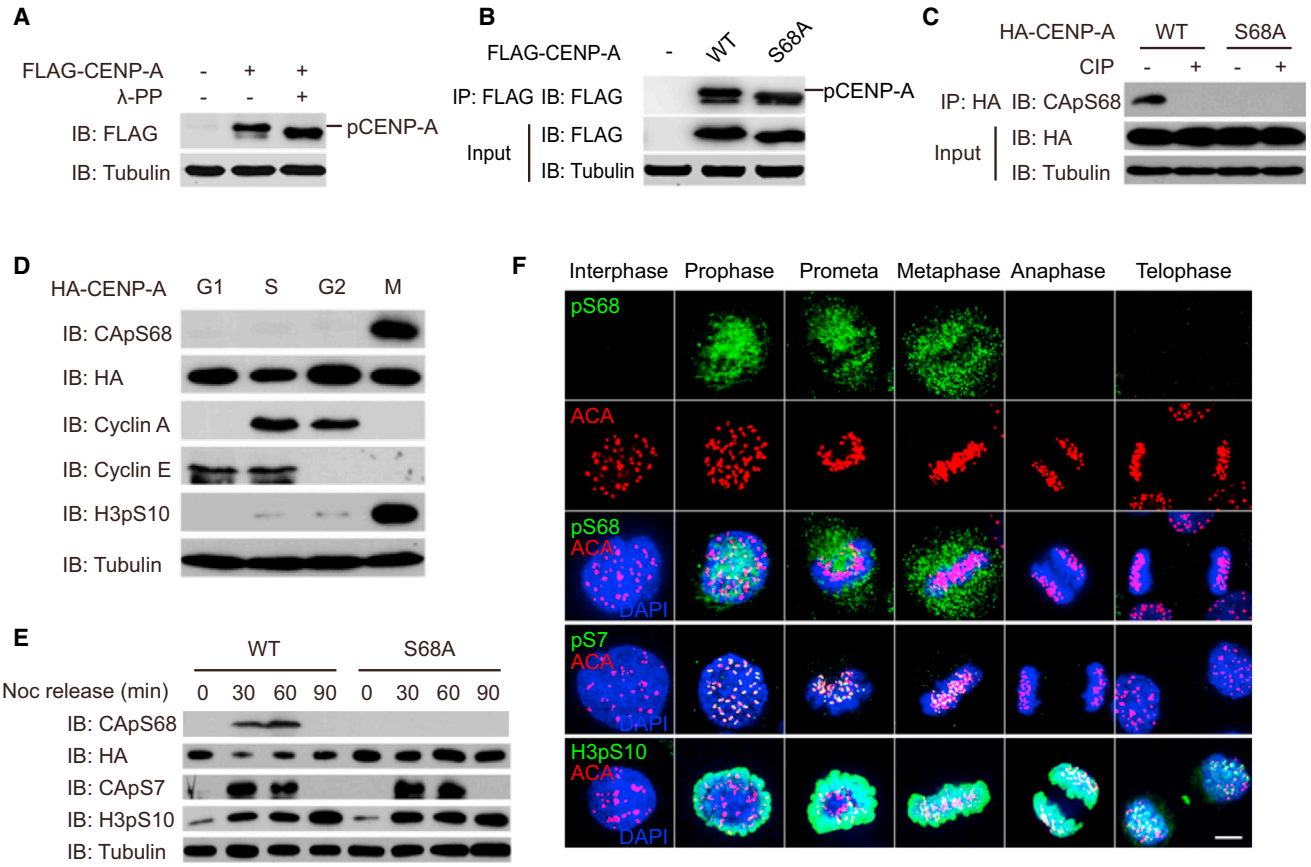

Figure 2. Ser68 Phosphorylation of CENP-A Occurs at the Early Mitotic Phase

(A) FLAG-tagged CENP-A was precipitated with anti-FLAG agarose from HEK293T cells transfected with FLAG-tagged CENP-A and was either mock-treated or treated with lambda PP, followed by western blot analysis using antibodies as indicated. Arrow indicates phosphorylated CENP-A. IB, immunoblot.

(B) HEK293T cells were transfected with either FLAG-tagged CENP-A or CENP-A^{S68A}. Whole-cell extracts were subjected to immunoprecipitation (IP) with anti-FLAG agarose and analyzed by western blotting using antibodies as indicated. Arrow indicates pSer68 CENP-A. WT, wild-type.

(C) HA-tagged CENP-A was precipitated with anti-HA agarose from HEK293T cells transfected with HA-tagged CENP-A or CENP-A^{S68A} and was either mock-treated or treated with calf intestinal alkaline phosphatase (CIP), followed by western blot analysis using antibodies as indicated.

(D) Western blot analysis of 293T cells expressing HA-tagged CENP-A in different cell-cycle stages; cyclin A, cyclin E, and H3pS10 immunoblots indicated specific stages, while α-tubulin immunoblot served as a loading control.

(E) Western blot analysis of 293T cells overexpressing HA-tagged CENP-A or CENP-A^{S68A} released from nocodazole (Noc) treatment for the indicated times; CENP-ApS7 and H3pS10 are mitotic markers, while α-Tubulin served as a loading control.

(F) Representative images of CENP-A Ser68 phosphorylation at mitotic phase. HeLa cells were stained with a phosphor-specific antibody against either Ser68 or Ser7 of CENP-A or Ser10 of H3 (pS68/pS7/H3pS10, green), together with ACA (for centromeres, red) and DAPI (for DNA, blue). Scale bar, 5 μm.

Interestingly, our results indicate that the Q68S mutation in H3.1^{CATD} not only facilitates binding to the chaperone HJURP (Figures 1J, 1K, and S2B–S2D) but also rescues the full spectrum of kinetochore-related functions of CENP-A in vivo (Figures S2E and S2F). Taken together, our results suggest that, apart from the essential role of CATD, CENP-A Ser68 is also absolutely required for the recognition of CENP-A by HJURP (Black et al., 2007; Cho and Harrison, 2011; Foltz et al., 2009; Hu et al., 2011; Zhou et al., 2011).

CENP-A Ser68 Is Phosphorylated in the Early Stages of Mitotic Phase

In light of the critical role of Ser68 for the function of CENP-A, we sought to investigate whether this site undergoes posttranslational modifications. Previous studies have demonstrated that CENP-A can be phosphorylated at Ser7, Ser17, and Ser19 (Bailey et al., 2013). Indeed, a slower migrating band of FLAG-CENP-A was observed when using the lysates of cells that overexpressed FLAG-CENP-A, and the band disappeared when the lysates were pretreated with lambda protein phosphatase (PP) (Figure 2A). To examine whether the Ser68 of CENP-A can be phosphorylated in vivo, we generated a CENP-A mutant in which the Ser68 residue was mutated to alanine (CENP-A^{S68A}) to render this site completely devoid of phosphorylation. Interestingly, a clear and specific shift-up of the CENP-A band was observed in the lysates of cells expressing wild-type CENP-A, relative to the CENP-A band from cells expressing the CENP-A^{S68A} mutant (Figure 2B), suggesting that Ser68 is phosphorylated in vivo. To further identify and characterize the role of Ser68 phosphorylation in CENP-A, we generated a site-specific anti-pSer68 antibody, which exhibited high specificity to the phosphorylated Ser68 (pSer68) in CENP-A in dot blot, western

blot, and immunofluorescent assays (Figures S3A–S3C). Using this antibody, we confirmed that the overexpressed CENP-A in 293T cells was phosphorylated at Ser68 (Figure 2C). Mutations of Ser68 to either alanine (S68A) or glutamate (S68E) not only eliminated the phosphorylation of this site but also resulted in the mislocalization of CENP-A into noncentromeric regions, indicating that phosphorylation of Ser68 is essential for the specific deposition of CENP-A into centromeres (Figure S3C). Interestingly, by using synchronized 293T cells, we also found that the phosphorylation of CENP-A at Ser68 primarily occurred during the mitotic phase of the cell cycle (Figure 2D). Notably, this phosphorylation could only be detected within a short time window after the entry into mitosis (most likely from prophase to metaphase) and became undetectable 90 min after the release from G_2-M arrest induced by nocodazole, just prior to exit from mitosis (Figure 2E). To accurately characterize the timing of Ser68 phosphorylation, we performed immunofluorescent staining experiments using our newly generated anti-pSer68 antibody. Our results showed that phosphorylation of Ser68 started in prophase, reached its maximal levels during prometaphase, and decreased during anaphase and telophase (Figure 2F). While this pattern occurred concurrently with the phosphorylation of CENP-A Ser7 (Figure 2F) (Kunitoku et al., 2003; Zeitlin et al., 2001), CENP-A^{pSer68} displayed a localization pattern that was very different from that of CENP-A^{pSer7}. At early mitosis, CENP-A^{pSer7} was localized specifically at centromeres, whereas CENP-A^{pSer68} was dispersed throughout the whole nuclear region in mitotic cells. Furthermore, the dispersed CENP-A^{pSer68} signal was found to merge neither with DAPI-stained chromatin nor with ACA-stained centromeres (Figure 2F). These results suggest that the phosphorylation of Ser68 occurs exclusively in prenucleosomal CENP-A and may prevent the deposition of CENP-A into centromeric regions. To verify this hypothesis, we performed cell fractionation experiments to separate the soluble CENP-A from the chromatin-bound CENP-A. The results clearly showed that phosphorylated Ser68 was only detected in free, prenucleosomal CENP-A but not in the chromatin-bound CENP-A (Figure S3D). In summary, our results reveal that CENP-A is phosphorylated at Ser68 during early mitosis before its recruitment to the centromere, which starts in the late mitotic phase.

Phosphorylation at Ser68 Regulates the Recognition of CENP-A by HJURP, a Vital Process for Centromere Integrity In Vivo

Based on our previous structural and biochemical results, we speculated that the Ser68 phosphogroup of CENP-A disrupts the recognition surface of CENP-A and HJURP due to steric hindrance issues (Figure 3A). We analyzed whether the phosphorylation of Ser68 or its phosphomimic mutation (S68E) affects the binding of CENP-A to HJURP using a molecular dynamics (MD) simulation study, a method that computationally simulates the physical interaction between molecules. Interestingly, as illustrated in Figure 3B, our simulation analysis revealed that both Ser68 phosphorylation and its phosphomimic mutant form significantly disrupt the recognition pocket interacting with HJURP (Figure 3B; Movie S1). Together with the results of our coIP experiments with HJURP and CENP-A mutants (shown in Figure S1), we speculated that the binding of CENP-A to HJURP might be inhibited by phosphorylation of Ser68 occurring during the early phase of mitosis. To experimentally verify this hypothesis, we examined the binding of endogenous CENP-A to HJURP at different phases of the cell cycle using synchronized cell populations. As predicted, significantly decreased binding of CENP-A to HJURP was observed in mitotic cells compared with cells in other phases (Figures 2D–2F and 3C). We used synchronized cells to compare the binding of ectopically expressed wild-type CENP-A to HJURP with that of the CENP-A^{S68A} mutant. Our results for samples of synchronized cells in the mitotic phase showed a significant decrease in HJURP binding of wild-type CENP-A at this cell-cycle stage but not for CENP-A^{S68A} mutant (Figure 3D). These results indicate that the phosphorylation of Ser68 indeed prevents the binding of CENP-A to HJURP. To further study the effect of Ser68 phosphorylation on the binding of CENP-A to HJURP, we used a phosphomimic mutant of CENP-A, S68E (CENP-A^{S68E}). Using coIP experiments, we showed that, similar to the CENP-A^{S68Q} mutant, the phosphomimic form CENP-A^{S68E} exhibited greatly impaired binding to HJURP (Figure 3E), confirming the inhibitory effect of Ser68 phosphorylation on CENP-A binding to HJURP. In contrast, replacement of serine with alanine in the CENP-A^{S68A} mutant resulted in HJURP binding affinity higher than that of wild-type (Figures 3D and 3E), indicating that the both charge and size of residue 68 are critical for the recognition of CENP-A by HJURP. Using LacI-*LacO* targeting assays, we found that the CENP-A^{S68E} mutation impaired the tethering of CENP-A to HJURP but not, however, to the histone H4-interacting chaperone RbAp46 (Figures 3G and 3H). RbAp46 was previously observed in the prenucleosomal CENP-A complexes in *Drosophila* (Furuyama et al., 2006) and human cells (Dunleavy et al., 2009; Hayashi et al., 2004). Intriguingly, in our coIP experiments, we found that HJURP only bound to unmodified CENP-A, while RbAp46 bound to both unmodified and pSer68 CENP-As (Figure 3F). Our in vitro supercoiling assays confirmed that RbAp46 equally assembled unmodified wild-type CENP-A and CENP-A^{S68Q} into a chromatin array, indicating that RbAp46 binds to phosphorylated CENP-A complexed with histone H4 during the mitotic phase (Figure S4A). Thus, inhibition of binding of CENP-A to HJURP by Ser68 phosphorylation might allow CENP-A to bind to chaperones RbAp46/48 along with H4.

Similar to the CENP-A^{S68Q} mutant, overexpression of CENP-A^{S68E} in HeLa cells resulted in the mislocalization of CENP-A and various mitotic chromosomal defects (Figures S4B and S4C). Because CENP-A^{pSer68} was found to bind to RbAp46 but not to HJURP, we hypothesized that RbAp46 might be the chaperone that mediates the mislocalization of CENP-A^{S68E} into noncentromeric regions. Indeed, through knockdown experiments using HJURP- and RbAp46/48-specific small hairpin RNAs (shRNAs), we found that HJURP was primarily responsible for the centromere-specific loading of CENP-A and dispensable for the deposition of CENP-A^{S68E} to noncentromeric regions (Figures 3I and 3J). By contrast, we noticed that the noncentromeric localization of CENP-A^{S68E} disappeared, while the centromeric CENP-A was not affected when RbAp46 and RbAp48 were silenced simultaneously (Figures 3I and 3J), suggesting that RbAp46/48 are responsible for the noncentromeric localization of CENP-A. A recent study reported that the noncentromeric localization of CENP-A is also mediated by the H3.3 chaperone

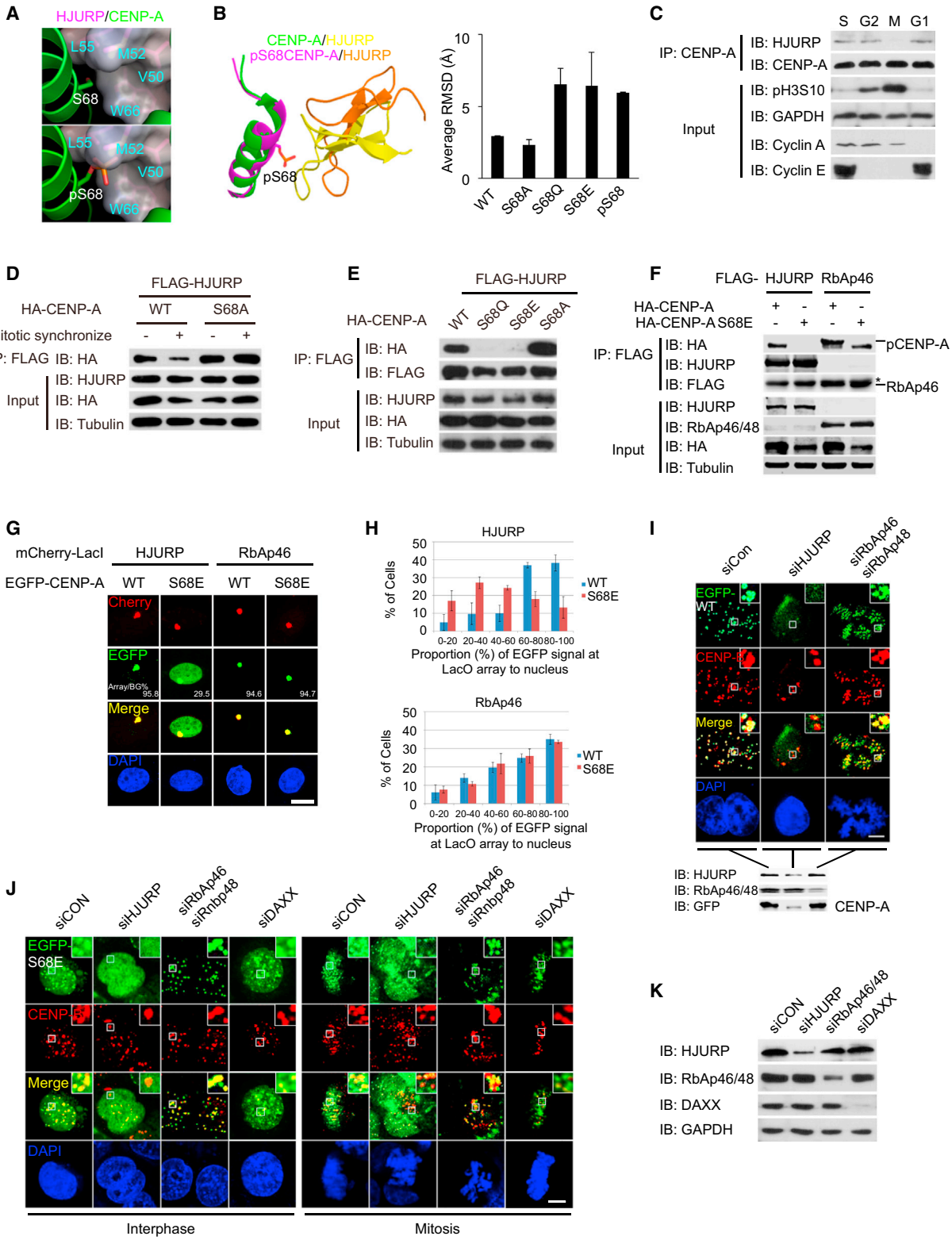

Figure 3. Phosphorylation of Ser68 Regulates the Recognition of CENP-A and Centromere Integrity

(A) Structure of HJURP/CENP-A (Protein Data Bank [PDB] ID 3R45) and proposed model for HJURP/pSer68 CENP-A interaction. HJURP is shown as a semitransparent surface superimposed with a stick model of key residues (carbon, magenta; nitrogen, blue; oxygen, red; sulfur, yellow). CENP-A Ser68 is shown as green sticks superimposed on the ribbon representation of the α1-helix. White and cyan letters label selected CENP-A and HJURP residues, respectively.

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DAXX (Lacoste et al., 2014). However, we found that DAXX was not the primary factor responsible for the mislocalization of CENP-A^{S68E} mutant (Figure 3J). Given the aforementioned results, we conclude that the phosphorylation of Ser68 plays an important role in regulating the recognition of CENP-A by the chaperone HJURP during the cell cycle and that HJURP-unbound CENP-A might be associated with other chaperones such as RbAp46/48 during mitotic phases of the cell cycle. Like DAXX, RbAp46/48 may be a noncentromeric assembly factor of CENP-A that contributes to the deposition of CENP-A in regions outside the centromere (Bodor et al., 2014).

In agreement with the effect of Ser68 phosphorylation on the recognition of CENP-A by chaperones, massive chromosomal defects were observed in cells overexpressing the Ser68 phosphomimic CENP-A mutants. In addition to centromeric deposition, CENPA^{S68E} displayed a promiscuous chromatic spreading during interphase and throughout mitosis (Figure 3J; Figure S3C), which was accompanied by the various mitotic chromosomal defects (Figures S4B and S4C). Taken together, our results strongly demonstrate that the phosphorylation of Ser68 plays an important role in regulating the recognition of CENP-A by chaperones and centromere integrity during cell cycle.

Cdk1/Cyclin B Is Required for Phosphorylation of CENP-A at Ser68

To identify potential kinases responsible for the phosphorylation of CENP-A at Ser68, we isolated CENP-A-associated proteins using a combination of affinity purification and mass spectrometry (Foltz et al., 2009). In addition to HJURP, we identified RbAp46/48, nucleophosmin 1, histone H4, and a number of kinases, including Cdk1 and Cdk2, in the prenucleosomal CENP-A complex (Figure S5A). Importantly, the interaction between Cdk1 and CENP-A was detected by coIP experiments (Figure S5B). Additionally, we performed a screening of known mitotic kinases by overexpressing various kinases, along with hemagglutinin (HA)-tagged CENP-A, in 293T cells. Of all kinases tested, only Cdk1 was found to induce a detectable increase of Ser68 phosphorylation of CENP-A (Figure 4A). Moreover, we found that roscovitine, a specific inhibitor of Cdk1 and Cdk2 activity, significantly reduced the phosphorylation of Ser68 in wild-type CENP-A-expressing 293T cells (Figure 4B). Together, our results suggest that Cdk1 might be responsible for the phosphorylation of Ser68 during the early mitotic phase, the exact time when Cdk1 is known to be activated. In support of Cdk1 as a bona fide kinase for Ser68, we observed that the phosphorylation of Ser68 was severely reduced after knockdown of Cdk1 by its specific shRNA (Figures 4C and 4E). In comparison, phosphorylation was not affected upon knockdown of Aurora A, a kinase responsible for CENP-A Ser7 phosphorylation (Kunitoku et al., 2003). Furthermore, a similar reduction of the phosphorylation of Ser68 was observed upon knockdown of cyclin B1, an essential component of the Cdk1 kinase complex (Figure 4D). More important, similar to exogenously overexpressed CENP-A, endogenous CENP-A was also phosphorylated by Cdk1/cyclin B in mitotic cells, as shown by both western blot (Figure 4E) and immunofluorescent staining assays (Figure 4F). Finally, Ser68 of CENP-A was specifically phosphorylated by Cdk1/cyclin B in an in vitro kinase assay (Figures 4G and S5C). Together, we concluded that Cdk1/cyclin B is responsible for the phosphorylation of Ser68, which occurs during early mitosis.

Ser68 Phosphorylation Is Necessary for the Regulation of CENP-A Loading onto Centromeric Chromatin

After establishing that HJURP binding of CENP-A is regulated by its Ser68, we reasoned that this specific phosphorylation event might play additional roles in controlling the deposition of newly imported CENP-A following DNA replication in order to complete the duplication of centromeres. To examine whether the phosphorylation of Ser68 perturbs the timing of newly synthesized CENP-A loading at defined phases of the cell cycle, quench-chase-pulse assays based on SNAP-tagging were performed (Jansen et al., 2007) (Figure 5A; Figure S6A). The SNAP-tagged wild-type CENP-A exhibited exclusive centromere-specific localization, comparable to that of EGFP-tagged wild-type CENP-A (Figures 1E and 1H; Figure S6B). In contrast, the SNAP-tagged mutant form CENP-A^{S68E} displayed severe

(B) Left: a superimposed structural representation for the conformational change introduced by Ser68 phosphorylation with HJURP. The conformation of CENP-A wild-type (WT) (green) and HJURP (yellow) are obtained from the crystal structure of CENP-A/H4/HJURP (PDB ID 3R45). Conformation of pSer68 CENP-A (magenta) and deformed HJURP (orange) is a representative snapshot from the MD simulation. Right: Quantification of structural deformation by CENP-A Ser68 mutants or phosphorylation as measured by the average root-mean-square deviation (RMSD) of the C-terminal part of HJURP segment in the last 50-ns snapshots of two independent MD simulations. Error bars indicate mean ± SD.

(C) Immunoprecipitation (IP) was performed using anti-CENP-A antibody to enrich endogenous CENP-A in synchronized cells in different phases, and HJURP binding was analyzed by western blot. H3pSer10, cyclin A, and cyclin E immunoblots (IBs) indicated specific cell phases.

(D) HEK293T cells were transfected with FLAG-tagged HJURP and HA-tagged CENP-A or CENP-AS68A, followed by immunoprecipitation with anti-FLAG agarose and analyzed by western blot using antibodies as indicated.

(E) The effects of different mutations at CENP-A Ser68 on HJURP binding. HEK293T cells were transfected with FLAG-tagged HJURP and HA-tagged CENP-A WT or Ser68 mutants, followed by immunoprecipitation analysis with anti-FLAG agarose and western blot analysis using antibodies as indicated.

(F) HEK293T cells were transfected with the FLAG-tagged HJURP or RbAp46, along with HA-tagged CENP-A or CENP-A^{S68E} (for Ser68 phosphorylation mimicry experiments). Whole-cell extracts were subjected to immunoprecipitation with anti-FLAG agarose followed by western blot analysis using antibodies as indicated.

(G) Representative images of A03_1 cells transfected with EGFP-tagged CENP-A or CENP-A^{S68E} mutant (green) along with mCherry-LacI-HJURP or mCherry-LacI-RbAp46 (red) and stained with DAPI (DNA, blue). Scale bar, 10 μm.

(H) Quantification of cells with EGFP-tagged proteins specifically enriched at *LacO* arrays in (G). The enrichment is defined by the ratio (%) of the signal on the array to that in the background (nucleus). Data are shown as mean ± SD of three independent experiments.

(I and J) The deposition of CENP-A upon knockdown of HJURP, RbAp46, or DAXX. HeLa cells were transfected with either EGFP-tagged CENP-A (I) or CENP-A^{S68E} (J) (green) along with shRNA plasmids as indicated. Cells were stained with anti-CENP-B (for centromeres, red) and DAPI (DNA, blue). Scale bar, 5 μm. Expression of endogenous proteins was analyzed by western blot (I and K).

(K) Western blot showing expression levels of endogenous HJURP, RbAp46/48, and DAXX in (J) after RNAi knockdown using antibodies as indicated.

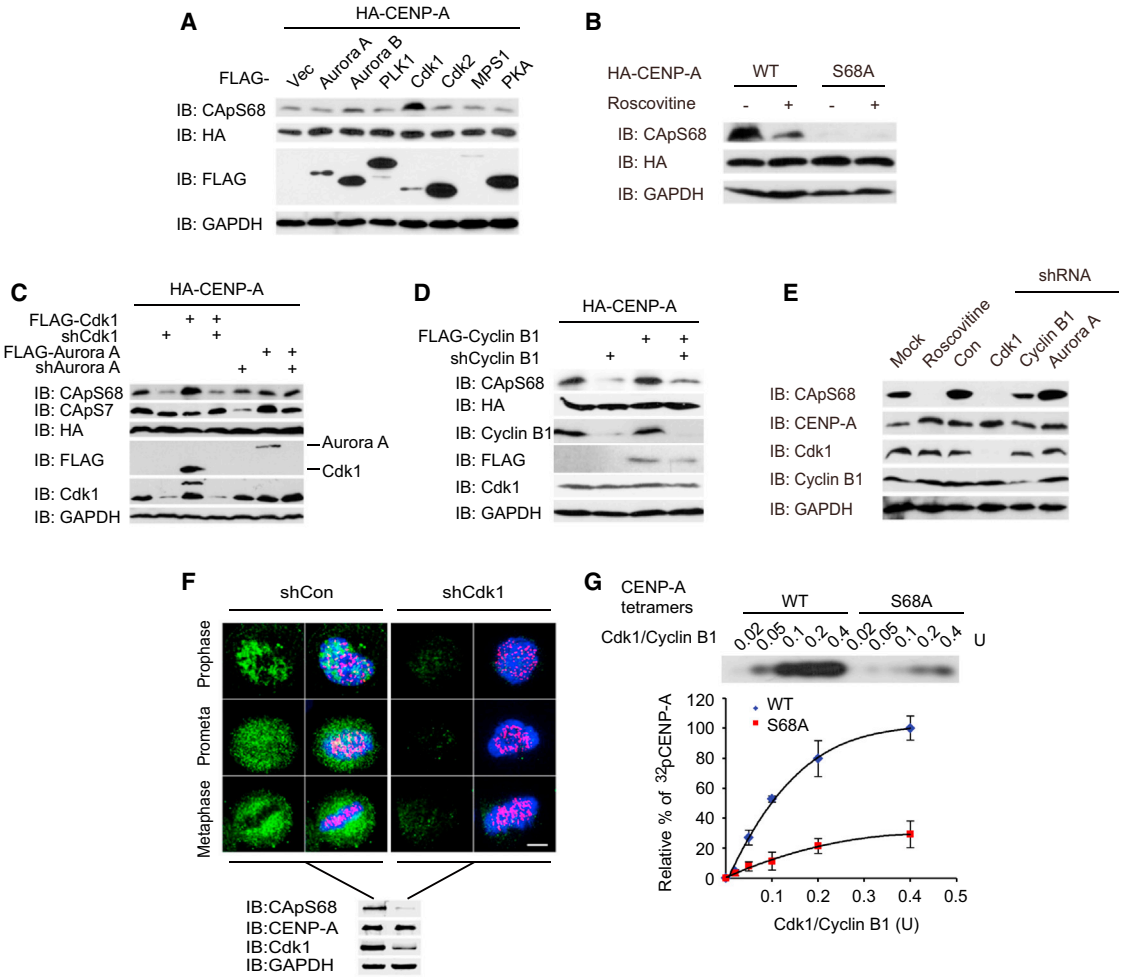

Figure 4. The Cdk1/Cyclin B Complex Is Required for the Phosphorylation of CENP-A Ser68

(A) A kinase screening to identify the potential kinase responsible for the Ser68 phosphorylation of CENP-A. HEK293T cells were transfected with HA-tagged CENP-A and FLAG-tagged kinases, followed by western blot using antibodies as indicated. IB, immunoblot. Plk1, polo-like kinase 1.

(B) Western blot analysis of Ser68 phosphorylation of CENP-A in inhibitor-treated cells. HEK293T cells were transfected with vectors overexpressing HA-tagged CENP-A wild-type (WT) or the S68A mutant, treated with or without roscovitine.

(C and D) HEK293T cells were transfected with vectors expressing FLAG-tagged proteins and/or shRNA plasmids, followed by western blot analysis using antibodies as indicated.

(E and F) Western blot (E) and immunofluorescence analysis (F) of Ser68 phosphorylation of endogenous CENP-A when Cdk1 was knocked down.

(G) In vitro kinase assay of CENP-A Ser68-phosphorylation. CENP-A wild-type or S68A tetramer were incubated with a series of different concentrations of Cdk1/cyclin B in the presence of [γ-^{32}P]ATP, followed by SDS-PAGE and autoradiography. Error bars indicate mean ± SD.

mislocalization across all chromosome regions, at both interphase and mitosis (Figure S6C). Although the S68A mutant does not have any significant effect on the binding of CENP-A to HJURP (Figures 3D and 3E), SNAP-CENP-A^{S68A} exhibited a different localization pattern compared with SNAP-tagged wild-type CENP-A. A portion of SNAP-tagged CENP-A^{S68A} was also shown to mislocalize at noncentromeric regions at interphase and the mitotic phase to a lesser extent compared to SNAP-tagged CENP-A^{S68E} (Figure S6C), suggesting that phosphorylation of CENP-A Ser68 might have another function in CENP-A dynamics in addition to its inhibitory effect on HJURP recognition. To analyze the role of Ser68 phosphorylation in regulating CENP-A assembly during the cell cycle, we used HeLa cells stably expressing SNAP-tagged wild-type CENP-A or its mutants (CENP-A^{S68E} and CENP-A^{S68A}). In these cells, exogenous CENP-A was expressed at levels similar to those of endogenous CENP-A, as shown by western blot (Figure 5B). HeLa cells were synchronized at the G_1-S boundary by double-thymidine block, and the preexisting SNAP-tagged CENP-A was quenched with nonfluorescent bromothenylpteridine blocking agent. The cells were then released into S phase and cultured for 7 hr, and newly synthesized SNAP-tagged CENP-A was pulse-labeled for 30 min with TMR-Star prior to fixation and assaying by immunofluorescent microscopy. SNAP-tagged CENP-A started to appear at centromeres ~11 hr after release from the double-thymidine block, which is concomitant with the exit of mitosis and entry into G_1 phase (Jansen et al., 2007). The expression of SNAP-tagged wild-type CENP-A was

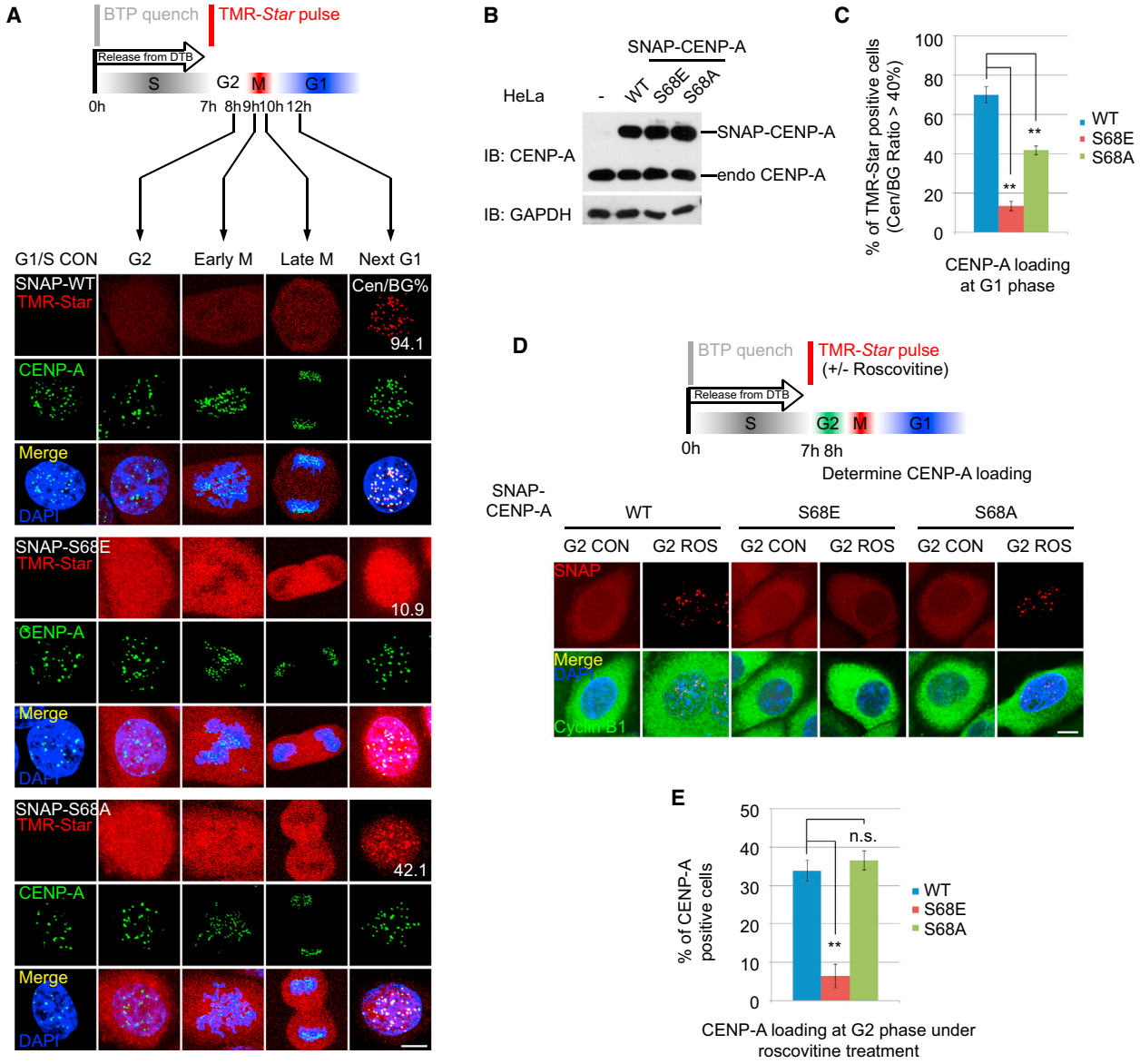

Figure 5. Ser68 Phosphorylation Regulates the Temporal Loading of Newly Synthesized CENP-A

(A) The loading of newly synthesized SNAP-tagged CENP-A wild-type (WT) or Ser68 mutants during the cell cycle. Upper panel: schematic diagram illustrating the outline of cell synchronization and labeling regimen for CENP-A loading. Lower panel: representative images of HeLa cells stably expressing SNAP-tagged wild-type CENP-A or mutants (red) were synchronized and labeled as depicted and stained with anti-CENP-A (for centromeres, green) and DAPI (for DNA, blue). Quantification data (ratio) of SNAP-CENP-A loading in G1 are listed next to the images. Scale bar, 5 μm.

(B) Western blot showed protein expressions of endogenous and SNAP-tagged CENP-A in (A).

(C) Quantification of cells with centromeric SNAP-CENP-A localization in (A). The SNAP-CENP-A-positive cells were defined by the ratio (%) of SNAP-TMR-Star signals at centromere to background signal (nucleus) as indicated. Data are presented as mean ± SD of three independent experiments. $^{**}p < 0.01$ (Student's t test).

(D) The premature loading of SNAP-CENP-A wild-type or Ser68 mutants in G_2 phase by treatment with roscovitine. Upper panel: schematic diagram illustrating the outline of roscovitine treatment and CENP-A labeling. Lower panel: representative images of HeLa cells stably expressing SNAP-tagged CENP-A or mutants (red) were synchronized and labeled as depicted and stained with anti-Cyclin B (for marker of G_2 phase, green) and DAPI (for DNA, blue).

(E) Quantification of cells in (D). Cells with clearly dispersed centromeric SNAP-TMR-Star signals are defined as CENP-A-positive cells. Data are presented as mean ± SD of three independent experiments. $^{**}p < 0.01$ (Student's t test), n.s.: no significant difference.

detectable at low levels at the G_2 phase, and very low levels of SNAP-tagged wild-type CENP-A were widely dispersed throughout the nuclear region throughout the M phase (Figure 5A). By contrast, significant levels of the SNAP-tagged CENP-A^{S68E} mutant started to accumulate and disperse in the nuclei shortly after pulse-labeling (at the G_2 phase), and they persisted throughout the entire time course of the assay (Figure 5A). Interestingly, none of the SNAP-tagged CENP-A^{S68E} mutant was

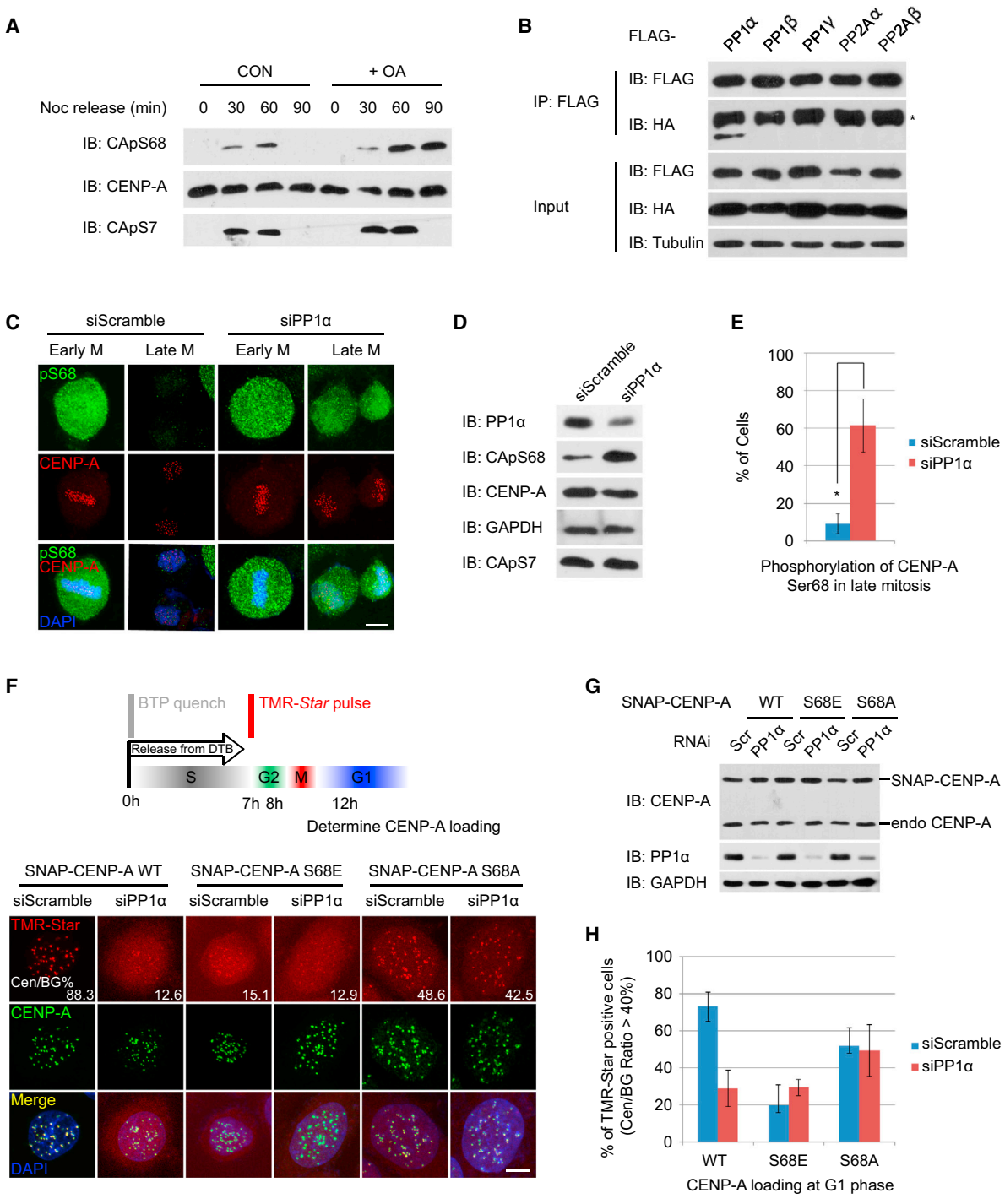

Figure 6. CENP-A Ser68 Is Dephosphorylated by PP1α during Late Mitosis

(A) Western blot analysis of CENP-A Ser68 phosphorylation by treatment with OA, a PP1/PP2A inhibitor. HEK293T cells were synchronized at G_2-M phase by nocodazole (Noc) and then released in the presence of OA. IB, immunoblot; Con, control.

(B) Western blot analysis of FLAG-immunoprecipitates (IPs) from HEK293T cells transfected with FLAG-tagged PP1/PP2A isoforms along with HA-tagged CENP-A. Asterisk indicates a nonspecific band.

(C) Representative images of mitotic cells illustrate Ser68 phosphorylation of HeLa cells transfected with shRNA plasmid that contain either scrambled or PP1α-knockdown shRNA. Cells were stained with a phosphospecific antibody against CENP-A Ser68 (pS68, green), together with anti-CENP-A (for centromeres, red) and DAPI (for DNA, blue). M, mitosis. Scale bar, 5 μm.

(D) Western blot analysis showing PP1α expression and Ser68 phosphorylation in (C) using antibodies as indicated.

(E) Quantification of late mitotic cells of pSer68 CENP-A in (C). *$p < 0.05$ (Student's t test). Error bars indicate mean ± SD.

(legend continued on next page)

shown to exhibit centromere-specific localization throughout the time course of the assay, indicating that this Ser68 phosphomimic mutant impairs HJURP binding of CENP-A and thus prevents the assembly of new CENP-A into the centromere. Similar to the SNAP-tagged CENP-A^{S68E} mutant, the SNAP-tagged CENP-A^{S68A} mutant also started to accumulate and disperse in nuclei of G_2 cells or nuclear regions during the M phase (Figure 5A). This observed accumulation beyond wild-type levels suggests that phosphorylation at Ser68 also plays an important role in controlling CENP-A protein stability during the cell cycle. In addition, portions of SNAP-tagged CENP-A^{S68A} were assembled into centromeres during mitotic exit and early G_1 phase, similar to wild-type SNAP-tagged CENP-A (Figure 5A). As mentioned earlier, we also found that small portions of newly synthesized SNAP-tagged CENP-A^{S68A} were assembled into noncentromeric regions during the cell cycle in our quench-chase-pulse assays. We also investigated the different mutants and their impact on premature CENP-A loading at G_2 phase by addition of roscovitine (Silva et al., 2012). As shown in Figures 5D and 5E, the newly synthesized wild-type CENP-A and CENP-A^{S68A} mutant forms, but not CENP-A^{S68E} mutant, could be prematurely loaded at the centromere by inhibition of Cdk1/2. These results indicate that only the unmodified CENP-A could be loaded into centromeres by HJURP upon release of centromeric chromatin licensing factors and chaperones, such as Mis18 complex and HJURP, by Cdk inhibition. Taken together, our results reveal that CENP-A Ser68 phosphorylation is critical for regulating the spatiotemporal deposition of CENP-A during cell division.

Dephosphorylation of Ser68 by PP1α Ensures Precise CENP-A Deposition during the Cell Cycle

As revealed here, the binding of CENP-A to HJURP is inhibited by the phosphorylation of Ser68. In order for HJURP to load the newly synthesized CENP-A into centromeric chromatin, the inhibitory phosphorylation of Ser68 must be removed prior to CENP-A deposition at the telophase/early G_1 phase transition. Therefore, we set out to identify the phosphatases that are responsible for the dephosphorylation of CENP-A Ser68. We found that the phosphorylation of Ser68 was increased by the treatment of HeLa cells with okadaic acid (OA), a Ser/Thr-PPs inhibitor that specifically inhibits the activity of PP1/PP2A family phosphatases (Cohen, 1989), suggesting that PP1/PP2A might be responsible for the dephosphorylation of Ser68 (Figure 6A). Indeed, a number of Ser/Thr PPs, including PP1α, PP1β, and PP2Aα, have been found in prenucleosomal CENP-A complexes (Figure S5A). To support this notion, we performed coIP experiments to verify the interactions between phosphatases and CENP-A using 293T cells transfected with plasmids expressing FLAG-tagged PP1/2A isoforms and HA-tagged CENP-A. Interestingly, our results showed that only PP1α, but none of the other phosphatases interacted with CENP-A, suggesting that CENP-A is a potential substrate of PP1α (Figure 6B). To investigate whether PP1α is responsible for the dephosphorylation of CENP-A at Ser68 during CENP-A deposition, we performed immunofluorescence assays. Knockdown of PP1α significantly increased the phosphorylation level of Ser68 during late mitosis, while in the control knockdown with scrambled shRNA, phosphorylation decreased greatly at telophase (Figures 6C–6E), indicating that PP1α dephosphorylates Ser68 and renders CENP-A available for deposition by HJURP at mitotic exit. In addition, as shown in quench-chase-pulse assays, knockdown of PP1α significantly impaired the centromeric deposition of wild-type CENP-A during G_1 phase, whereas the deposition of the S68A mutant was not affected (Figures 6F–6H). Taken together, the results provide strong support for the notion that PP1α is the phosphatase responsible for the dephosphorylation of CENP-A at Ser68 and is required for the temporal deposition of CENP-A during the cell cycle.

DISCUSSION

Proper spatiotemporal assembly of CENP-A into active centromeres must be tightly controlled during the cell cycle in order to ensure faithful chromosome segregation and the maintenance of genome integrity (Black and Cleveland, 2011). In summary, as illustrated in Figure 7, our present study demonstrates that newly synthesized CENP-A is immediately phosphorylated at Ser68 by Cdk1/cyclin B at G_2 and the early mitotic phases and that this particular phosphorylation impairs the binding to HJURP, thus preventing the premature incorporation of CENP-A into centromeres. As CENP-A deposition occurs, the phosphorylation at Ser68 is removed by PP1α to ensure that CENP-A is available for deposition by HJURP.

Ser68 Is a Phosphorylation Switch Site Essential for the Recognition of CENP-A by HJURP

CENP-A deposition is a chaperone-based chromatin assembly event that specifically occurs at centromeres (Gurard-Levin et al., 2014). In this study, using biochemical and cell-based assays, we demonstrated that, apart from the CATD of CENP-A, Ser68 serves as an important regulatory site for the recognition of CENP-A by HJURP through cell-cycle-dependent reversible phosphorylation, which is consistent with our previous structural results (Hu et al., 2011). In direct contradiction to our results, Black and colleagues, using a similar cell-based LacI-*LacO* chromosome-targeting approach, showed that the S68Q mutation has no detectable effect on the binding of CENP-A to HJURP (Bassett et al., 2012). At the same time, however, their hydrogen-deuterium exchange analysis validated the interaction between the C-terminal β sheet region of the HJURP histone binding domain and the α1-helix of CENP-A, which is in agreement

(F) Upper panel: schematic diagram illustrated the outline of synchronization and labeling of SNAP-CENP-A. Lower panel: representative images showed the deposition of newly synthesized SNAP-CENP-A (TMR-Star) in HeLa cells stably expressing SNAP-tagged CENP-A wild-type or mutants transfected with plasmid contains either scramble shRNA or PP1α shRNA. Cells were stained with anti-CENP-A antibody for centromere (green). Scale bar, 5 μm.

(G) Western blot showing protein expressions of endogenous and SNAP-tagged CENP-A of cells in (F).

(H) Quantification of cells with centromeric SNAP-CENP-A (TMR-Star) localization in (F). The SNAP-CENP-A-positive cells are defined by the ratio (%) of SNAP-TMR-Star signals at centromere to the SNAP-TMR-Star signals in the background (nucleus) as indicated. Data are presented as mean ± SD of three independent experiments.

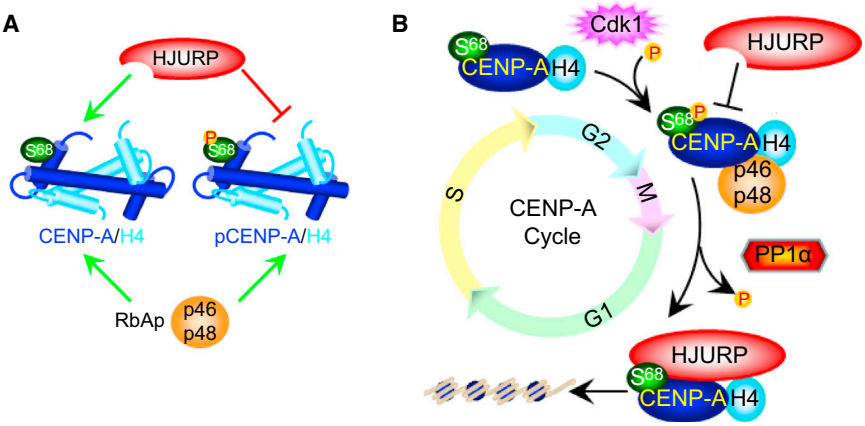

Figure 7. Proposed Model for the Spatiotemporal Regulation of CENP-A Deposition through the Equilibrium between Phosphorylation and Dephosphorylation at Ser68 by Cdk1/Cyclin B and PP1α during the Cell Cycle

(A) A simplified cartoon illustrating that the binding activities of CENP-A to different chaperones are regulated by CENP-A Ser68 phosphorylation.

(B) Schematic diagram depicting regulation of CENP-A deposition through Ser68 phosphorylation during the cell cycle.

with our present and previous studies (Bassett et al., 2012; Hu et al., 2011). The different results from the two very similar cell-based LacI-*LacO* targeting assays might be due to the different systems used to visualize the overexpressed CENP-A. In our study, we directly visualized the EGFP-tagged CENP-A in living cells under relatively natural conditions, whereas the other study performed indirect immunofluorescence under relatively harsh conditions for preparing their samples. Thus, the chromatin-unbound CENP-A(s) (especially the S68Q mutant) was possibly washed out and may, therefore, have become undetectable in their indirect immunofluorescence experiments. Only CENP-A targeted to either chromatin or HJURP-bound *LacO* arrays can be detected in their cell-based approach, which may be the primary reason why no effect was observed for the S68Q mutant form on the binding of HJURP in their LacI-*LacO* targeting assays. Indeed, similar to our previous study, the phenomenon of mistargeting observed for the S68Q mutant was visibly alleviated when we used an indirect immunofluorescence approach to visualize either HA- or EGFP-tagged CENP-A and its S68Q mutant in the LacI-*LacO* targeting assays (Figure S7). Thus, a two-site mechanism is proposed for the recognition of CENP-A by HJURP, with CATD providing the main binding region and with the reversible phosphorylation at Ser68 providing an additional regulatory site essential for HJURP recognition.

The Dynamic Phosphoregulation of Ser68 Regulates the Spatiotemporal Deposition of CENP-A into Centromeric Chromatin by Regulating CENP-A Interaction with HJURP

Recently, Silva et al. (2012) demonstrated that the machinery for CENP-A assembly is present and poised by Cdks prior to mitosis. Furthermore, inhibition of Cdks activities resulted in rapid premature centromeric CENP-A assembly in a manner that is dependent on the CENP-A assembly machinery that assembles CENP-A into centromeric chromatin, which is accompanied by rapid recruitment of Mis18α and M18BP1 to the centromeres (Silva et al., 2012). Strikingly, they found that the unscheduled targeting of the phosphorylation-dead mutant of Mis18BP1^{HsKNL2} (Mis18BP1$^{HsKNL2\text{-}Ala24}$) did not result in premature centromeric CENP-A assembly (Silva et al., 2012). These results indicate that the Cdk-dependent phosphorylation-mediated inhibition of CENP-A assembly is likely exerted through controlling the centromeric localization of key factors or the machinery for CENP-A assembly. Moreover, several recent studies have demonstrated that the centromeric chromatin licensing via phosphorylation of the Mis18 complex by polo-like kinase 1, as well as the inhibition of premature centromeric localization of HJURP and Mis18 complex through phosphorylation by Cdk1, also contribute to the temporal control of CENP-A deposition (McKinley and Cheeseman, 2014; Müller et al., 2014; Wang et al., 2014). Thus, a sophisticated regulatory phosphorylation network appears to play a paramount role in the CENP-A deposition pathway.

In summary, our results showed that Cdk1 mediates the phosphorylation of CENP-A at Ser68 and inhibits the binding of CENP-A to HJURP to prevent premature targeting of CENP-A to the centromeres during early mitosis. Thus we propose that, in addition to the scheduled "priming of CENP-A deposition" by the Mis18 complex and HJURP, the dynamic phosphorylation/dephosphorylation of Ser68 in CENP-A, which is mediated by the Cdk1/cyclin B and PP1α complex, is another important mechanism that temporally controls the HJURP-mediated assembly of CENP-A into centromeric regions. Together with previous studies, our results presented here strongly support the idea that the dynamic phosphorylation/dephosphorylation of CENP-A Ser68 by Cdk1 and PP1α, together with specific chaperones (Gurard-Levin et al., 2014; Müller et al., 2014) and the "priming" factors (McKinley and Cheeseman, 2014; Müller and Almouzni, 2014), may provide an on/off-switch mode that orchestrates the spatiotemporal assembly of CENP-A at centromeres. Therefore, for faithful delivery of genetic information across generations, CENP-A propagation, as part of centromere inheritance, has evolved under the tight control of cell-cycle-dependent phosphoregulatory networks, which represent the primary regulatory force that ensures the proper progress through the cell cycle.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Protein Preparation

All plasmids used in this study were constructed following standard molecular biology techniques. The expression and purification of proteins are described in detail in the Supplemental Experimental Procedures.

Cell Culture and Synchronization

Human embryonic kidney 293T (HEK293T) and HeLa cells were cultured under standard conditions. For cell-cycle-dependent analysis, cells were blocked by

either double-thymidine or nocodazole treatment for G_1-S and G_2-M arrest, respectively. See Supplemental Experimental Procedures for details.

SNAP-Pulse Labeling

HeLa cells stably expressing SNAP-tagged CENP-A wild-type or mutants were synchronized at the G1-S boundary. SNAP-tag was quenched with SNAP-Cell Block (New England Biolabs, S9106S), after which cells were released into S phase. Newly synthesized CENP-A–SNAP was labeled 7 hr after release with SNAP-Cell TMR-Star (NEB, S9105S). Cells were fixed at the indicated time courses and processed for CENP-A assembly by microscopy.

Immunoprecipitation and Immunofluorescence

Stable cell lines of HEK293T and HeLa cells transfected with plasmids as indicated were treated and harvested at various cell stages. Protein-protein interaction and protein localization were analyzed by immunoprecipitation and immunofluorescence, respectively. For fluorescent microscopy, cells were seeded on glass coverslips, washed in PBS, and fixed in PBS with 0.05% Tween 20 (PBST) containing 4% paraformaldehyde for 15 min at room temperature. After three washes in PBST, cells were permeabilized in PBST-0.1% Triton X-100 buffer for 15 min, blocked in PBST containing 5% BSA for 30 min, and then incubated with primary antibodies overnight at 4°C. Cells were washed with PBST and incubated with fluorescence-conjugated secondary antibodies at 37°C for 1 hr. Cells were stained for DNA with 10 μg/ml DAPI (Sigma, D9542) in PBST for 15 min before mounting with SlowFade Gold antifading reagent (Invitrogen, P36939). As shown in Figure S7, immunofluorescence was performed as previously reported (Bassett et al., 2012). See Supplemental Experimental Procedures for details.

Western Blotting and Microscopy

Western blotting was performed following standard procedures. Immunofluorescent images were collected on a Zeiss Axio Observer Z1 microscope (Carl Zeiss MicroImaging). Antibodies (See Supplemental Experimental Procedures) suitable for western blotting or immunofluorescence were used as indicated.

Chromatin Fractionation

Chromatin fractionation was performed as described previously (Kang et al., 2011). For the fractionation of HA-tagged CENP-A or CENP-A^{S68Q}-expressing HEK293T cells, equal volumes of each fraction were analyzed by western blotting. For the investigation of the phosphorylation of CENP-A Ser68, the loading volumes of all samples were adjusted to keep the total amount of CENP-A identical for each lane. For detailed information, see Supplemental Experimental Procedures.

In Vitro Chromatin Assembly Assays

The in vitro chromatin assembly assay was performed as described previously (Barnhart et al., 2011). See Supplemental Experimental Procedures for details.

In Vitro Kinase Assays

For the in vitro kinase assay, 2 μg of the wild-type or S68A mutant CENP-A-H4 tetramer was mixed with 0.01–1 U Cdk1/cyclin B (NEB, P6020) in 1× PK buffer. Reaction was initiated by the addition of ATP to a final concentration of 500 μM with 1 μM [γ-32P]ATP (PerkinElmer, NEG002, 10 Ci/mmol). After 1 hr of incubation at 30°C, the reaction was terminated by addition of SDS loading buffer, prior to analysis by SDS-PAGE and autoradiography.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2014.11.030.

AUTHOR CONTRIBUTIONS

Z. Yu and X. Zhou carried out the experiments, analyzed the data, and composed the figures. W.W. instigated the project and assisted with the construction of plasmids and the generation of stable cell lines. W.D. performed the TAP purification of CENP-A-associated proteins and validated the phosphor-antibody by peptides. J.F. and H.H. assisted with the expression of recombinant proteins and the construction of plasmids. Z.W. performed the in vitro chromatin assembly assays. S.L. and X. Zhang constructed the FLAG-HJURP knockin cell line. L.C. and J.L. performed the MD simulation. J.S. assisted with the western blot analysis. L.Z. and P.X. performed the mass spectrometric analysis. J.W. and S.D. constructed the A03_1 cell line that containing tandem *LacO* repeats. S.P. and Z. Yuan assisted with the knockdown experiments. G.O. assisted with the fluorescent microscopy experiments. N.Y. and P.C. assisted with helpful discussions and revised the manuscript. G.L. and R.-M.X. conceived of and supervised the project. G.L. designed and supervised the experiments, analyzed the data, and wrote the manuscript.

ACKNOWLEDGMENTS

We are grateful to Dr. Hong Zhang and Dr. Bing Zhu for critical reading and discussion of our manuscript. We thank Dr. T. Juelich for linguistic assistance and discussions during the preparation of this manuscript. This work was supported by grants from the Ministry of Science and Technology (2011CB966300 to G.L.), the National Natural Science Foundation of China (91219202 and 91019007 to G.L., 31210103914 to R.-M.X., and 31000566 to P.C.), and the Chinese Academy of Sciences Strategic Priority Research Program (XDA01010304 to G.L.) and Key Research Program (KSZD-EW-Z-009 to P.C. and N.Y.). All fluorescence imaging data were collected and processed at the Center for Bio-imaging, Core Facility for Protein Sciences, Institute of Biophysics, Chinese Academy of Sciences. The computational resources for the MD simulations were provided by the Supercomputing Center of the Chinese Academy of Sciences and the National Supercomputing Center Tianjin Center. The mass spectrometry analysis was performed at the Core Facility for Protein Sciences, Institute of Biophysics, Chinese Academy of Sciences.

Received: March 29, 2014
Revised: October 14, 2014
Accepted: November 19, 2014
Published: December 31, 2014

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